

Antimicrobial and Cytotoxic Activities of Endophytic Fungi Isolated from *Artemisia annua* L

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ABSTRACT

This study was aimed to investigate antimicrobial and cytotoxic effects of endophytic fungi isolated from *A. annua* L. The ethyl acetate extracts were obtained by liquid-liquid partition of fermentation broth of endophytes followed by evaporation. Antimicrobial assay was determined by disc diffusion method. Cytotoxicity assay was conducted against T47D, WiDr and Vero using MTT assay. Data generated were used to determine IC₅₀. While DP2 extract did not show any inhibition against all tested bacteria, DP6 extract at 200 µg inhibited the growth of *B. subtilis*, *P. aeruginosa* and *S. thypi* with the highest inhibition against *B. subtilis*. Similarly, E2 extract inhibited the growth of *B. subtilis*, *E. coli* and *S. thypi*, with the highest inhibition against *B. subtilis*. The three extracts (DP2, DP6 and E2) were found to be cytotoxic against T47D and WiDr with E2 extract to be the most cytotoxic. These compounds were more selective against T47D cell line with IC₅₀ of 91.7, 74.8 and 12.0 µg/mL, respectively. The IC₅₀ against Vero cell line are 444.4, 140.3 and 87.7 µg/mL, respectively.

INTRODUCTION

Natural products are sources of bioactive compounds potential to be developed as pharmaceutical agents. Endophytic microbes are one group of microorganisms capable of producing active metabolites which then attract the attention of scientists since the finding of paclitaxel which can be generated by a fungus colonizes Himalayan yew tree, *Pestalotiopsis microspore* (Tan and Zou, 2001). Endophytic microbes reside in living plant tissues without causing damage to the host. They colonize in plant tissues such as in leave, stem, root and seed and have been recognized to produce bioactive metabolites potentially having medicinal values such as alkaloids, terpenoids, polyketides, phenylpropanoids, aliphatic compounds, and peptides (Pinheiro *et al.*, 2013; Mousa *et al.*, 2013; Zheng *et al.*, 2013; de Souza *et al.*, 2011). *Artemisia annua* L., is a plant which grows in tropical countries and known to produce artemisinin with a range of concentration of 0.01-1.1 % (Alam *et al.*, 2010). Artemisinin is a famous drug against malaria, one of the most deadly infections in tropical countries. This compound and its derivatives have been reported to have such

anticancer activities with various mechanisms of actions as cell cycle arrest, apoptosis, inhibition of angiogenesis, inhibition of Wnt/β-catenin pathway and disruption of cell migration (Fireston *et al.*, 2009; Krishna *et al.*, 2008; Li, 2007; Zhou, 2007). However, the low level of production of artemisinins from the *A. annua*, limits its exploration directly from the plants for commercialization. Other methods are developed to find bioactive compounds from *A. annua*. Drimartol A, a sesquiterpenecoumarin has been isolated from *A. annua* hairy root culture and this compound was reported to have anticancer activity (Zhai and Zhong, 2010). Endophytes of *A. annua* are also a source of microorganisms potential to be developed for production of bioactive compounds. Twenty one of 39 endophytes of *A. annua*, have been isolated and reported to have antifungal activities (Liu *et al.*, 2001). Artemisinin, the antimalarial drug has been reported to be produced by endophytic actinobacteria *Pseudonocardia* sp (Li *et al.*, 2012). Many studies reported the ability of antimicrobial compounds to function as anticancer agents and anthracyclines are an example of a well-known antibiotic used as anticancer drugs (Mordente *et al.*, 2012). In this study we investigated the antimicrobial and cytotoxic activities of compounds produced by endophytes of *A. Annua* collected from National Centre for Research and Development of Medicinal Plants and Traditional Medicine, Tawangmangu, Indonesia.

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MATERIALS AND METHODS

Source of endophytic fungi

Plant materials were collected from National Centre for Research and Development of Medicinal Plants and Traditional Medicine, Tawangmangu, Indonesia. The parts of plant were collected and stored at 4° C until used. The living plant was grown on green house, the species was identified by Faculty of Biology Universitas Gadjah Mada Indonesia and the specimen was stored in the Faculty of Pharmacy Universitas Gadjah Mada Indonesia.

Isolation of endophytic fungi.

Isolation of endophytic fungi from *A. annua* L, was carried out by sterilizing the surface of healthy plant tissues in running tap water for 10 minutes. After washing with 70% ethyl alcohol for 1 min, the samples were soaked in 5% sodium hypochlorite for 3 min, immersed with 70% ethyl alcohol for 30 s and followed by thrice rinses with sterile distilled water.

The surfaces sterilized samples were cut aseptically into small pieces of 1 cm long segments using a sterile blade and placed on the plates with PDA medium supplemented with streptomycin (30 µg/mL) for incubation at 25 °C. Each fungal colonies growing in plant tissues were transferred into new PDA plates without antibiotics until cultures were obtained for fermentation and collection. The culture collections were deposited in Pharmaceutical Biology Department, Faculty of Pharmacy UniversitasGadjahMada, Indonesia.

Semipolar extraction of fermentation cultures

The pure fungal endophytic cultures were fermented in 200 mL culture flasks containing potato dextrose broth for 10 days at 25 °C on a shaker at 160 rpm. Following fermentation, the culture broth were filtered with Whatman filter paper and centrifuged at 4 000 rpm for 5 min. Liquid supernatant was extracted with an equal volume of ethyl acetate thrice and the ethyl acetate fractions were evaporated to yield an ethyl acetate extract. The extracts were dissolved in ethanol for antimicrobial and cytotoxicity testing.

Antimicrobial activity

Screening for antimicrobial activities was carried out by using disc diffusion method (Momin *et al.*, 2014) against four pathogenic bacterial strains (*B. subtilis*, *E.coli*, *P. aeruginosa*, *S. typhi*) at dose of extracts of 200 µg/disc. The test microorganisms were inoculated in 10 mL sterile nutrient agar and transferred into petri dishes in aseptic condition to give a solid plate. The extracts were applied into the disc and deposited on the surface of agar plates. The plates were incubated overnight at 37 ° C. Clear zones of inhibition surrounding the disks were measured at the end of incubation and the diameters were expressed in mm.

Cytotoxic activity

Three cell lines used in this study were T47D (Human ductal breast epithelial tumor cell line), WiDr (human colon

carcinoma cell line) and Vero (African green monkey kidney epithelial cell line). The cells were cultured in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin and maintained at 37°C in an atmosphere of 5% CO₂. Cytotoxicity assay conducted by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay adapted from Mosmann (1983) with minor modification. Control and treated cells inoculated at 96 well plate were incubated for 24 h and read using a spectrophotometer at 595 nm. The concentration of extracts required to kill 50% of cell population (IC₅₀) was determined from data generated by plotting a dose-response curve.

RESULTS AND DISCUSSION

Endophytic Fungi and Its Metabolite Characterization

Total of seven distinct fungal isolates were obtained from the plant collected from National Centre for Research and Development of Medicinal Plants and Traditional Medicine, Tawangmangu, Indonesia. Based on initial screening, three of them (namely DP2, DP6 and E2) produced compounds having antibacterial activities. The chromatographic profiles of the extract under UV 366 and UV 254 indicated the presence of various compounds within the extracts (Figure 1). There was one major compound within DP6 with hRf of 0.40 which absorb 254 nm UV light to give dark spots against green fluorescence.

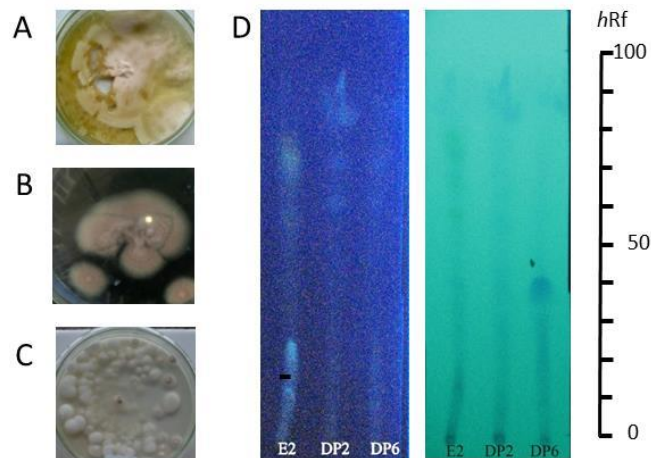


Fig. 1: Morphology characteristics of endophytic fungi isolated from *A. annua* and TLC profiles of its ethyl acetate extracts. Endophytic fungi DP2 (A), DP6 (B) and E2 (C) grown on PDA; its TLC profiles (stationary phase: silica gel F₂₅₄; mobile phase: chloroform: ethyl acetate: 1:2) detected using UV₃₆₆ and UV₂₅₄ (D).

Antimicrobial activities

Ethyl acetate extracts obtained from the fermentation broth of the three fungi were screened for antimicrobial activities against *B. subtilis*, *E.coli*, *P. aeruginosa*, *S. typhi* (Table 1). Two extracts at given concentration (200 µg) showed any inhibition against testing microorganisms. DP6 and E2 extracts showed greater activities against *B. subtilis*. These extracts also inhibited the growth of *S. typhi*, although the inhibition zone is less than that

against *B. subtilis*. In addition, DP6 inhibited the growth of *P. aeruginosa*, whilst E2 against *E. coli*. DP2 extract at dose of 200 µg did not show any inhibition against four testing microorganisms.

Table 1: Antimicrobial activity of extracts against *B. subtilis*, *E. coli*, *P. aeruginosa*, *S. typhi* as determined by disc diffusion test.

No	Extracts (200 µg)	Inhibition zones (mm)			
		<i>B. subtilis</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. typhi</i>
1	DP2	-	-	-	-
2	DP6	24	-	7.08	6.87
3	E2	20.85	9.50	-	10.35

Cytotoxic activity

An attempt to examine the bioactivity of these three compounds against cancer cell lines was conducted. Cytotoxicity of the extracts against T47D and WiDr cell lines is shown in Table 2. At dose of 250 µg/mL addition of DP6 extract showed more death than DP2. In addition less dose were needed to kill T47D cells upon administration of E2 extract (Figure 2). Generally, the extracts were found to be more effective against T47D than the WiDr cell line. IC₅₀s of these compounds < 100 µg/mL against T47D and one of them (E2) had IC₅₀ of < 20 µg/mL. These compounds were found to be less cytotoxic against WiDr, with IC₅₀ values of DP2, DP6 and E2 of 332.9, 128.9 and 79.8 µg/mL, respectively.

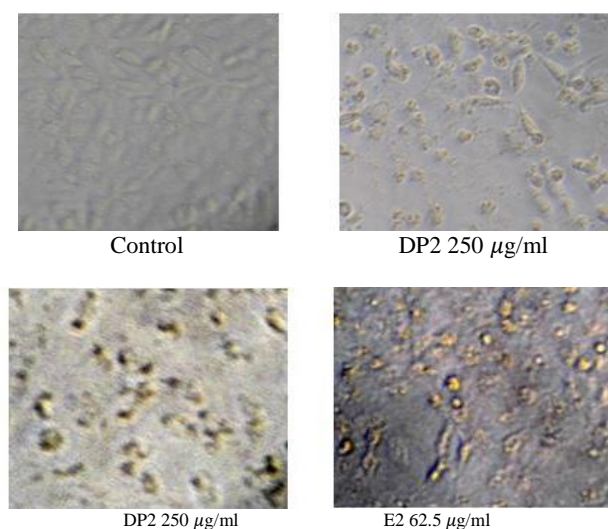


Fig. 2: Morphologic profiles of T47D cells treated with ethyl acetate extracts of DP2, DP6 and E2 at given concentration.

Table 2: IC₅₀ of endophytic extracts against T47D, WiDr and Vero cell lines (n = 4)

Extracts	IC ₅₀ (µg/mL)		
	T47D	WiDr	Vero
DP2	91.7	332.9	444.4
DP6	74.8	128.9	140.3
E2	12.0	79.8	87.7

To examine the selectivity of these compounds, cytotoxicity testing was also conducted against normal Vero cell line. We found that DP2, DP6 and E2 had IC₅₀ of 444.4, 140.3, 87.7 µg/mL, respectively. These results showed that the extracts were more

selective against T47D than WiDr. *A. annua* is a subtropical plant which is known to produce well known anti-malarial compound artemisinin. Beside its function as antimalarial drug, artemisinin and its derivatives have been reported to affect cancer cells with various mechanisms such as cell cycle arrest, apoptosis, disruption of cell migration as well as angiogenesis inhibition (Firestone and Sundar, 2009; Krishna *et al.*, 2008). On the other hand, endophytic fungi has been known to produce novel secondary metabolites with some of them possess bioactivities and medical values (Ally *et al.*, 2010; Katoch *et al.*, 2014). Li *et al.* (2012) reported the production of artemisinin by endophytic actinobacteria *Pseudonocardia sp.*

In agreement with the fact that fungal endophytes are potential sources of bioactive compounds, ethyl acetate extracts obtained from fermentation broth of endophytic fungi isolated from *A. annua* also showed bioactivities. Based on initial screening using hole plate assay, three from seven fungi isolated from *A. annua* collected from National Centre for Research and Development of Medicinal Plants and Traditional Medicine, Tawangmangu, Indonesia showed inhibition zones against testing microorganism. In this study the endophytes present in this plant may not all grow and have been isolated since some may need special conditions which were different with those in laboratory. The three endophytes then were further characterized for their potencies.

In this study we found that bioactivities of these three endophytes were not identical although they were isolated from the same species and the same locations. Only two of them showed inhibition against testing microorganisms with different level of inhibitions. Similarly, the bioactive abilities of endophytic fungi against cancer cell lines were not identical with one to be more cytotoxic than others; one to be more selective than others. These might be explained by the fact that the mixture compounds produced by these endophytes are different as characterized by TLC profiles.

Strongest inhibitions were clear against *B. subtilis*, whilst in general they were found to be more cytotoxic and selective against T47D. We found that one of the extract (E2) had IC₅₀ value of less than 20 µg/mL, the value within the standard point set up by National Cancer Institute for cytotoxicity screening of crude plant extracts (Lee *et al.*, 2005).

Many studies reported the ability of antimicrobial compounds to function as anticancer agents and anthracyclines are an example of a well-known antibiotics used as anticancer drugs (Mordente *et al.*, 2012). These data demonstrated that the extracts were potential to be developed as anti-cancer drugs and needs further investigation.

CONCLUSION

This finding suggested the presence of bioactive compounds within ethyl acetate extracts of endophytic fungi isolated from *A. annua* which are potential for development as pharmaceutical agent.

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